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Is transketolase-like protein, TKTL1, transketolase?

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ABSTRACT

Until recently it was assumed that the transketolase-like protein (TKTL1) detected in the tumor tissue, is catalytically active mutant form of human transketolase (hTKT). Human TKT shares 61% sequence identity with TKTL1. And the two proteins are 77% homologous at the amino acid level. The major difference is the absence of 38 amino acid residues in the N-terminal region of TKTL1. Site-specific mutagenesis was used for modifying hTKT gene; the resulting construct had a 114-bp deletion corresponding to a deletion of 38 amino acid residues in hTKT protein. Wild type hTKT and mutant variant (DhTKT) were expressed in *Escherichia coli* and isolated using Ni-agarose affinity chromatography. We have demonstrated here that DhTKT is devoid of transketolase activity and lacks bound thiamine diphosphate (ThDP). In view of these results, it is unlikely that TKTL1 may be a ThDP-dependent protein capable of catalyzing the transketolase reaction, as hypothesized previously.

1. Introduction

Transketolase (TKT, EC 2.2.1.1), a thiamine diphosphate (ThDP)-dependent enzyme, catalyzes C–C bond cleavage in ketoses (donor substrates) and the subsequent transfer of the resulting two-carbon fragment (glycoaldehyde residue) to aldoses (acceptor substrates) [1–4]. The enzyme is widely distributed in nature. The best-studied enzyme is TKT from *Saccharomyces cerevisiae* [3–6]. Transketolase from human tissues (hTKT) has been studied little. Until recently, the interest in TKT from human tissues was limited by medical implications (related to studies of neurodegenerative diseases) [7–10]. In 1996 Coy et al. first described transketolase-like protein, TKTL1, and suggested that it may have an altered or reduced transketolase activity [11]. Quite recently, transketolase-like protein, TKTL1, was found in the tumor tissue, which is considered to be a catalytically active mutant form of hTKT [12].[1] This is considered to be quite widely accepted, although no direct experimental evidence of this was found in the literature. It was shown that TKTL1 differs from hTKT in the primary structure, with sequence homology being 77% [16].

A computer model of the 3D structure of TKTL1 has been constructed using the known spatial structure of hTKT, and a structural comparison of the two proteins at all levels of their organization has also been performed [17]. On the basis of the revealed differences in structures of these proteins together with the fact that TKTL1 lacks an extended peptide fragment (38 amino acid residues) in the coenzyme-binding region, which is present, with minor modifications, in all ThDP-dependent enzymes characterized thus far [18], we could assume, it is unlikely that TKTL1 may be a ThDP-dependent protein capable of catalyzing the transketolase reaction. However, the final conclusion can be drawn only after additional, experimental evidence. The possibility that TKTL1 binds ThDP via an alternative mechanism cannot be ruled out. Examples of that kind are known, including those among ThDP-dependent enzymes. It is a well established fact, that the conserved glutamate, present in active centers of ThDP-dependent enzymes, is mandatory for enzymatic activity—thiamine catalysis is switched on by the interaction of this residue with N1′ atom of ThDP [19]. However, there is no such residue in the active center of glyoxylate carboxylase (it is substituted for by valine), which is still performing its catalytic function [20,21].

In this work, we sought to confirm (or to disprove) the conclusion made in the previous paper [17], based on the results of computer modeling, that TKTL1 is not a ThDP-dependent enzyme. To validate this conclusion experimentally, the deletion was introduced into the hTKT gene in order to obtain a variant analogous to TKTL1 which then could be used to examine its enzymatic reactivity. This mutant is missing the peptide of 38 amino acid residues in the N-terminal region of the protein (DhTKT)—the absence of which in TKTL1 mainly distinguishes this protein from hTKT [12,16].

Abbreviations: TKT, transketolase; hTKT, transketolase of normal human tissue; DhTKT, deletion mutant of hTKT; TKTL1, transketolase-like protein 1; ThDP, thiamine diphosphate

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[1] The presence of TKTL1 in tumor tissue has been used as an early diagnostic marker in progressing tumors since 2005 [12–15].

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2. Materials and methods

2.1. Chemicals

Chemicals used in this work included the following materials (grouped by the manufacturer): NAD^+, glyceraldehyde-3-phosphate dehydrogenase from rabbit muscle, ribose 5-phosphate and ThDP (Sigma Aldrich Chemie GmbH, Germany); dithiothreitol and $MgCl_2$ (Fluka, Germany); Bacto agar, Bacto tryptone and yeast extract ("DIFCO", USA); Ni-NTA-agarose ("Invitrogen", Germany); restriction endonucleases *Nco*I and *Nde*I, T4 DNA ligase and *Pfu* polymerase ("Fermentas", Lithuania); *Escherichia coli* strains ER1821 ("NEB", USA) and BL21 AI ("Invitrogen", Germany). Oligodeoxyribonucleotides 18-hTKTl (ATGGAGAGCTACCACAAG), 18-dhTt (AAACAAGCTTTCACCGAC) and p17-dhTb (pCTTGGAGAGCACAAAGC) were synthesized by Syntol (Russia). Plasmid pDEST-17/hTKT, containing hTKT gene, cloned in pDEST-17 expression vector ("Invitrogen", Germany), was kindly gifted by Dr. Johannes Coy (TAVARLIN AG, Germany). Xylulose 5-phosphate was synthesized as described previously [22]; the preparation obtained was 88% pure and contained no aldehyde admixtures. All genetic engineering manipulations were carried out using standard methods [23].

2.2. Measurement of protein concentrations

Concentration of hTKT was measured spectrophotometrically according to the value of the absorption coefficient of hTKT $A^{1\%}_{1\ cm} = 8.0$ at 280 nm, calculated by us from its amino acid composition using ProtParam software [24] (the same coefficient we used in the determination of DhTKT concentration).

2.3. Measurement of ThDP content in the preparations of hTKT and DhTKT

ThDP was separated from holo-hTKT using two approaches: heat treatment [1] and acid denaturation. The second approach consisted in adding perchloric acid (to the final concentration of 5%) to 25–100 μg hTKT or DhTKT in 0.1 ml 50 mM glycylglycine (pH 7.6). The mixture was incubated on ice for 15 min and centrifuged, after which the supernatant was collected and pH was adjusted to 6.5 using 1 M KOH. After 5 min, the mixture was centrifuged again and the supernatant was collected.

The concentration of ThDP in the supernatants, obtained by either of two approaches, was measured enzymatically by the method of Datta and Racker [1].

2.4. Activity measurements

Transketolase activity was measured by the rate of NAD^+ reduction, using glyceraldehyde-3-phosphate dehydrogenase as an ancillary enzyme and xylulose 5-phosphate/ribose 5-phosphate as substrates [3]. The reaction mixture had the following composition (final volume, 1 ml): 50 mM glycylglycine, 10 mM sodium arsenate, 0.37 mM NAD^+, 3 U glyceraldehyde-3-phosphate dehydrogenase, 3.2 mM dithiothreitol, 2.5 mM $MgCl_2$, 0.2 mM ThDP, 0.25–1.0 mM xylulose 5-phosphate, 0.5–2.0 mM ribose 5-phosphate, and 0.25–1.0 μg hTKT or 25–100 μg DhTKT (pH 7.6). The reaction was initiated by adding the substrates. Measurements were taken in 1-cm cuvettes at 340 nm, using a DW 2000 Aminco (USA) spectrophotometer.

2.5. Subcloning of hTKT gene fragment, mutagenesis and construction of expression vector for DhTKT

The NdeI/NcoI-fragment of pDEST-17/hTKT plasmid, corresponding to 5′-terminus of hTKT gene (498 bp) was subcloned into auxiliary vector pHNN (1691 bp plasmid, containing pUC19 origin of replication, β-lactamase operon, multiple-cloning site NdeI/NcoI) at NdeI and NcoI sites. The plasmid obtained was used as a template for whole-plasmid PCR mutagenesis [25] to generate modified hTKT gene fragment with target 114 bp deletion. The PCR reaction was carried out with the use of primers 18-dhTt and p17-dhTb and Pfu polymerase (12 cycles: 94 °C, 30 s; 54 °C, 20 s; 72 °C, 120 s). The PCR product obtained was circularized by T4 DNA ligase and then was used for transformation of the *E. coli* ER1821 cells. The structure of plasmid DNA of the clones in the region of NdeI/NcoI-insert was confirmed by sequencing using primer 18-hTKTl. NdeI/NcoI-fragment of initial pDEST-17/hTKT plasmid was replaced by modified NdeI/NcoI-fragment (384 bp) of auxiliary plasmid yielding pDEST-17/DhTKT expression plasmid. The structure of the gene, encoding hTKT with deletion (DhTKT), was confirmed by sequencing.

2.6. Expression and isolation of recombinant proteins

Recombinant hTKT of normal human tissues was obtained as described previously [26] using heterologous constitutive expression of protein in *E. coli* BL21 AI, transformed with pDEST-17/hTKT plasmid. Homogeneity of the protein was demonstrated by electrophoresis in 8% SDS-polyacrylamide gel (Fig. 1). Gel was stained with PageBlue™ Protein Staining Solution ("Fermentas", Lithuania). The specific activity of the protein was 5.3 U/mg at 20 °C.

Isolation of recombinant DhTKT was performed according to the protocol described for hTKT preparation, using heterologous constitutive expression in *E. coli* BL21 AI, transformed with pDEST-17/DhTKT plasmid, and Ni-NTA-agarose affinity purification. The homogeneity of preparation was checked by electrophoresis (Fig. 1). The excised Coomassie-stained protein bands generated from the SDS-PAGE were subjected to trypsin in-gel hydrolysis as described in [27]. Mass-spectra were obtained using MALDI-TOF mass spectrometer Ultraflex II BRUKER (Germany) equipped with a UV laser (Nd) (the mode of positive ions in the range of masses from 500 to 8000 Da). Mass-spectra were calibrated using known masses of internal standards. The identification of proteins was carried out by a peptide fingerprint search using Mascot software (Matrix Science, USA) through the NCBI protein database.

3. Results

3.1. Construction and expression of deletion variant of transketolase DhTKT

The hTKT gene devoid of 114 bp fragment encoding 38 amino acids fragment (positions 76–113) of the protein was engineered by PCR amplification and a series of cloning steps described in Materials and methods. A pDEST-17/DhTKT plasmid obtained was used for expression in *E. coli* BL21 AI the deletion variant of hTKT (DhTKT). His_6-tagged DhTKT was isolated from cytoplasm fraction using affinity

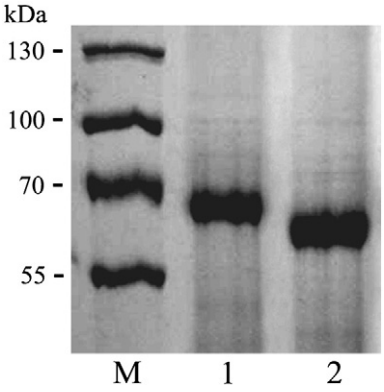

Fig. 1. Electrophoregram of hTKT and DhTKT. M—markers, 1—hTKT, 2—DhTKT. Gel was stained with PageBlue™ Protein Staining Solution.

chromatography on Ni-NTA-agarose. In contrast to *w.t.* hTKT protein mutant variant DhTKT was found mostly in inclusion bodies. From the cytoplasm of the cells it was possible to obtain no more than 0.1–0.2 mg of DhTKT per liter of stationary culture.

To evaluate the effect of 38 amino acid deletion on enzymatic activity a series of in vitro assays was conducted.

3.2. Measurement of DhTKT transketolase activity

Fig. 2 (in this case one of reproducible experiments is resulted) shows the determination of DhTKT transketolase activity and, to control—of hTKT. It is evident that for hTKT there is proportional dependence between the amount of the enzyme added to the sample (0.5–1.0 μg protein) and the reaction rate. Even after the addition of 25–100 μg of protein DhTKT to the reaction mixture and increasing the concentration of ThDP and substrates of transketolase reaction to 4-fold (compared to the conditions for following the activity of hTKT), it was not possible to register transketolase activity of DhTKT.

3.3. Measurement of ThDP content in the preparations of hTKT and DhTKT

Transketolase of normal human tissue is usually isolated in the form of holoenzyme and does not require addition of cofactors for developing of catalytic activity. Therefore at the next step it was necessary to identify the presence of ThDP in complex with proteins isolated. For separation of ThDP from holo-hTKT we used two well known approaches—heat treatment and acid denaturation, and for its identification and quantitative determination—specific and sensitive enzymatic method [1]. Two preparations of holo-hTKT and of DhTKT were analyzed, using both methods of ThDP separation (4 determinations of ThDP in every case). Both methods showed approximately the same results (Table 1)—about 1.0 molecule of ThDP per molecule of protein was determined. A different amount of bound ThDP in holo-hTKT correlates with the specific activity of the protein [26].

When we used the same approach of protein treatment to cleave ThDP from DhTKT and its subsequent determination, ThDP was not found. The result of this experiment demonstrated that DhTKT contained no ThDP.

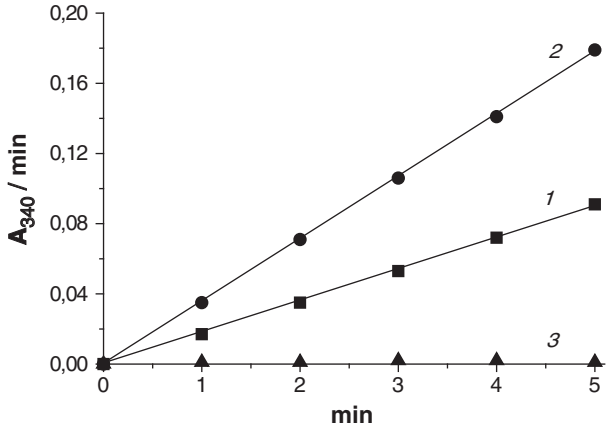

Fig. 2. Measurement of transketolase activity of DhTKT and hTKT. *1*—0.5 μg hTKT, 2—1.0 μg hTKT, *3*—100 μg DhTKT. The reaction mixture had the following composition (final volume, 1 ml): 50 mM glycylglycine, 10 mM sodium arsenate, 0.37 mM NAD^+, 3 U glyceraldehyde-3-phosphate dehydrogenase, 3.2 mM dithiothreitol, 2.5 mM $MgCl_2$, 0.2 mM ThDP, 0.25–1.0 mM xylulose 5-phosphate, 0.5–2.0 mM ribose 5-phosphate, and 0.5–1.0 μg hTKT or 100 μg DhTKT (pH 7.6).

Table 1
Amount of ThDP in preparations of holo-hTKT and DhTKT.

Protein	Number of ThDP molecules per molecule of protein	
Holo-hTKT	0.83 ± 0.10	1.25 ± 0.12
DhTKT	0	0

4. Discussion

It is considered that tumor tissue is characterized by increased activity of TKT [28]; most of (up to 85%) ribose required for the synthesis of nucleic acids in cancer cells is believed to be formed through the concerted action of hTKT and transaldolase [29]. Assuming that hTKT may constitute an adequate target for antitumor drugs, the search for hTKT inhibitors has received much recent attention; ongoing studies in this field largely focus on analogs of ThDP, the coenzyme of TKT [30,31].

In 2005, a TKTL1 protein was identified in human tumor tissue, which was assumed to represent a mutant form of hTKT [12], exhibiting TKT activity. Should this be the case, it would be justified to search for antitumor drugs among compounds targeting the mutant form, TKTL1, rather than hTKT previously known. However, no direct experimental evidence confirming the enzymatic transketolase activity of TKTL1 has been obtained thus far. To the best of our knowledge, there is only one report, in which the authors characterize a recombinant TKTL1 preparation as a native protein exhibiting transketolase activity [12]. This conclusion is far from being convincing, because the report contains neither general characteristics of the proteins obtained, nor protocols of their preparation. Moreover, the report lacks experimental curves of the kinetics of transketolase reaction (the figure included into the publication shows only relative enzymatic activity, but it is unclear what serves as the base for percentage calculation), the composition of the reaction mixture and the conditions under which the measurements were taken. We were unable to find in the literature any independent assessment of the conclusions drawn by the authors of the aforementioned report, which would provide direct experimental evidence that TKTL1 exhibits transketolase activity.

Our experimental data indicate that the deletion mutant of TKT from normal human tissues (DhTKT), lacking 38 amino acid residues (the presence of this deletion is the major feature distinguishing TKTL1 from hTKT), is incapable of catalyzing TKT reaction. This finding corroborates our prior assumption [17] that TKTL1 is distinct from the ThDP-dependent enzyme TKT. Our conclusion, therefore, is that the function of TKTL1 and its role, if any, in neoplasia, remain an open question.

Acknowledgements

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